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Loss of Function of MULTICOPY SUPPRESSOR OF IRA 1 Produces Nonviable Parthenogenetic Embryos in *Arabidopsis*

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Summary

In sexually reproducing species, fertilization brings together in the zygote the genomes of the female and male gametes. In several animal species, female gametes are able to initiate embryogenesis in the absence of fertilization, a process referred to as parthenogenesis. Parthenogenesis has been engineered in mice by tampering with expression of loci under epigenetic controls [1]. In plants, embryo development in the absence of fertilization has been reported in cases in which meiosis is bypassed leading to apomictic development, and parthenogenetic development from a reduced egg cell has been only reported in rare accidental cases [2]. We report that single mutations in the gene *MULTICOPY SUPPRESSOR OF IRA 1* (*MSI1*) are able to initiate parthenogenetic development of the embryo in *Arabidopsis thaliana* from eggs cells produced by meiosis. The WD40 repeat protein MSI1 is part of the evolutionarily conserved *Polycomb* group (PcG) chromatin-remodeling complexes [3] and is homologous to the Retinoblastoma binding proteins P55 in *Drosophila* and RbAp48 in mammals [4]. Nonviable haploid parthenogenetic *msi1* embryos express molecular markers and polarity similar to diploid wild-type (wt) embryos produced by fertilization, indicating a maternal contribution to early patterning of the *Arabidopsis* embryo.

Results and Discussion

Characterization of an Embryo-like Structure in the Mutant *msi1*

In the ovule of *Arabidopsis*, meiosis produces four haploid megaspores and is followed by the selection of one female gamete progenitor cell, which further undergoes three rounds of mitosis leading to the embryo sac [5]. The embryo sac contains two female gametes, the egg cell and the central cell. Two male gametes delivered by the pollen tube fertilize the two female gametes, leading to the development of the embryo from the egg cell and of the endosperm from the central cell. In some species, embryos are produced in absence of fertilization from unreduced egg cells, a process referred to as apomixis [2]. Unreduced egg cells often derive from cells in the ovule distinct from the megaspores. There have been a few reports in barley and in *Hieracium* of embryos produced without fertilization from a reduced egg cell, which derives from a megaspore. Such rare cases can be referred to as parthenogenetic embryogenesis [6, 7], and the molecular mechanism involved is not known. In contrast, autonomous development of endosperm from a reduced central cell has been found in several *Arabidopsis* mutants and is associated to the loss of function of the *Polycomb* group complex encoded by members of the *FERTILISATION INDEPENDENT SEED* (*FIS*) gene class [8–11]. Proliferation of the egg cell and the central cell has also been observed in loss-of-function mutant for the Retinoblastoma ortholog RBR1 [12]. Autonomous seeds produced by unfertilized *fis* mutant ovules do not contain an embryo, although rare cases of development of an embryo-like structure have been reported in some alleles of *medea* and of *fis2* [11] but not in other alleles [10, 13]. MSI1 is a putative Retinoblastoma binding protein and was recently identified as a member of the FIS complex [3, 4] and belongs to the *FIS* genetic pathway [13]. Autonomous seeds produced by emasculated heterozygous plants that carry *msi1-1* contain a syncytial tissue similar to endosperm, and a small cellular structure at the location of the embryo also develops where endosperm becomes initially cellular in wild-type fertilized seeds [3]. This structure was eventually considered as a cellularized derivative of endosperm [3]. Alternatively, this structure could derive from the egg cell. We undertook a detailed cytological and molecular characterization of this component in the autonomous *msi1* seeds in order to establish its developmental origin and identity.

In fertilized *Arabidopsis* wild-type seeds, parallel to the first divisions in the central cell, the zygote elongates and divides at 1.5 Days Post Anthesis (DPA) (n = 45). This is followed by a series of patterned divisions leading to an apical round embryo hooked to the maternal tissue by a filamentous suspensor (Figure S1A available with this article online). In absence of fertilization, the *msi1* egg cell undergoes elongation (Figures 1A and 1B) and divides at 3 DPA (Figure 1C; n = 56). Further divisions follow with randomly oriented planes of division until a small embryo-like structure with up to 20 cells is formed (Figures S1B and S1C; Figure 1D) and dies when the endosperm and the seed collapse between 8 to 10 DPA. Such embryo-like structure is observed in 90% of autonomous seeds (observation at 4.5 DPA; n = 245). Our cytological observations of autonomous *msi1* seeds led us to hypothesize that like the fertilized embryo sac, the unfertilized *msi1* embryo sac develops two different structures, which resemble an endosperm and an embryo-like structure. These would derive from the central cell and the egg cell, respectively. These observations indicate that an embryo develops autonomously from the unfertilized *msi1* egg cells.

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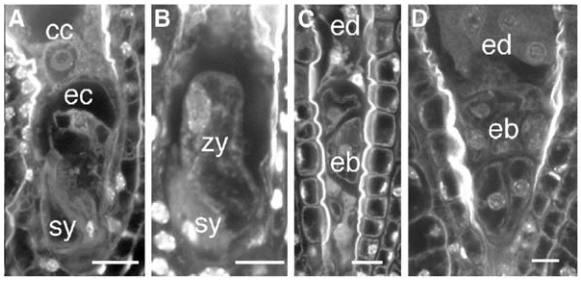

Figure 1. Confocal Sections of *msi1* Parthenogenetic Embryos at Different Times after Emasculation

(A) Undeveloped ovule. cc, central cell. ec, egg cell.
(B) At 2.5 DPA, one synergid degenerates (sy), and the egg cell elongates to form a zygote-like elongating cell (zy).
(C) At 3 DPA, the first divisions have occurred and give rise to an embryo-like structure (eb). Three nuclei of autonomous endosperm are visible (ed).
(D) A multicellular embryo-like structure is formed at 8 DPA. Scale bars, 10 μm.

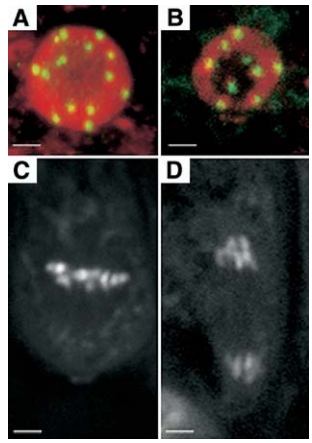

Figure 2. Ploidy Determination in *msi1* Autonomous Endosperm and Parthenogenetic Embryo

(A and B) Immunolocalization of centromeres in fertilized (A) and *msi1* autonomous (B) endosperm. Pictures result from the projection of successive confocal sections. DNA was stained by propidium iodide (red), and centromeres were immunodetected with anti-HTR12 antibody (green). Fifteen centromeres can be counted in a triploid fertilized endosperm nucleus (A), whereas nine centromeres are visible in this autonomous endosperm nucleus (B).
(C and D) Chromosome complements in embryonic mitotic figures after Feulgen staining. Pictures were obtained by superimposition of two confocal sections of mitotic figures. (C) Metaphase plate in a diploid fertilized embryo. Nine chromosomes are visible in the diploid nucleus. (D) Anaphase in a *msi1* parthenogenetic embryo. Five chromatids can be counted in the top complement and four chromatids are visible in the bottom complement. Scale bars, 2 μm.

Parthenogenetic Origin of the Embryo-like Structure

In the wt and in *msi1/MSI1* plants, the embryo sac develops from the meiotically reduced haploid megaspore and contains a haploid egg cell (n = 5) and a homodiploid central cell (2N = 10). In fertilized wt seeds, the embryo is diploid (2N = 10) and the endosperm is triploid (3N = 15). In order to determine the origin of the autonomous endosperm and embryo-like structure produced by *msi1/MSI1* plants in the absence of fertilization, we measure their ploidy. At mitosis, nuclei in autonomous *msi1* endosperm contain ten chromosomes (n = 24; data not shown). Accordingly, interphasic nuclei in autonomous *msi1* endosperm contain ten centromeres (n = 42) in comparison to 15 centromeres in fertilized wt endosperm (n = 62) (Figures 2A and 2B). This indicates that *msi1* autonomous endosperm is diploid because it develops from the unfertilized diploid central cell. If the cellularized structure at the anterior pole also derived from the autonomous endosperm, it should be diploid (n = 10). However, in the embryo-like structure dividing cells, we identify five chromosomes at metaphase (Figure S2L; n = 5) and two pools of five sister chromatids in separating nuclei during anaphase (Figure 2D; n = 12) in comparison to the dividing wt zygote that contains ten chromosomes (Figure 2C and Figure S2K). We conclude that the embryo-like structure in *msi1* autonomous seeds is haploid and likely derives from the egg cell as suggested by cytological observations.

The haploid nature of the embryo-like structure and the observation of successive elongation and division of the *msi1* egg cell strongly support a parthenogenetic development of the *msi1* egg cell into an embryo. However, haploid parthenogenetic *msi1* embryos undergo a limited number of divisions and do not show a wt embryo pattern at 8 DPA (Figure 1D), which questions their developmental identity. In order to ascertain the developmental identity of the structures, we tested the expression of endosperm and embryo molecular markers in autonomous endosperm and parthenogenetic embryos in *msi1* autonomous seeds. Five markers specific to the endosperm in fertilized WT seeds are also expressed in the autonomous *msi1* endosperm (Figures 3A and 3B, Figures S2A–S2H, and Table 1). Hence, the autonomous endosperm follows at least in part a developmental pathway typical of endosperm after fertilization. None of the endosperm markers are expressed in parthenogenetic *msi1* embryos (Figure 3B, Figures S2A–S2H, and Table 1). In contrast, parthenogenetic *msi1* embryos express typical markers of early wt embryos, PIN7-GUS [14] (Figures 3C and 3D; n = 102 embryos observed with expression of the marker) and G222 (Figures S2I and S2J; n = 119 embryos observed with expression of the marker). The absence of expression of endosperm markers and the expression of the early embryo markers in the parthenogenetic product of the egg cell indicate its embryonic identity. However, development of the parthenogenetic *msi1* embryo is limited to a small series of cell division leading to a misshapen mass of cells. This phenotype is observed to a lesser degree in diploid homozygous *msi1* embryos [13]. Because MSI1 probably controls cell proliferation via its interaction with the Retinoblastoma pathway [3], it is likely that both parthenogenetic haploid *msi1* and fertilized diploid *msi1/msi1* embryos arrest as a result from deregulation of the MSI1/Retinoblastoma pathway. Partial *msi1* autonomous endosperm development may also impair proper embryogenesis.

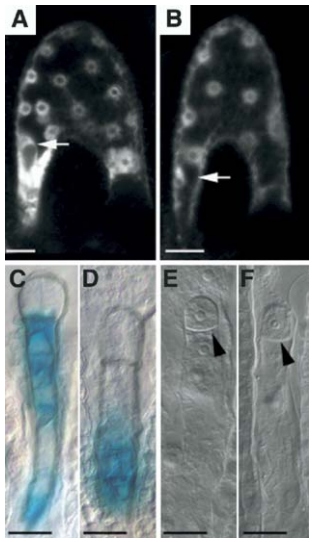

Figure 3. Expression of Endosperm and Embryo Markers in Fertilized and *msi1* Autonomous Seeds

(A) The enhancer-trap N9319 GFP marker is specifically expressed in endosperm but not in the embryo (arrow) after fertilization.

(B) At 3 DPA, the autonomous *msi1* endosperm expresses the N9319 GFP marker. A region at the anterior pole remains dark and does not express the GFP marker (arrow). This corresponds to the location of the parthenogenetic embryo.

(C) GUS staining (blue) reveals PIN7-GUS enhancer-trap line expression in a wt fertilized two-cell embryo (borders of the embryo cells are outlined).

(D) At 5 DPA, the two-cell *msi1-2* parthenogenetic embryo expresses PIN7-GUS in the basal cell (borders of the embryo cells are outlined).

(E and F) The first division in both fertilized (E) and *msi1* parthenogenetic (F) zygotes is apical and perpendicular to the apical-basal axis (arrowheads). (A and B) Scale bars, 100 μm. (C–F) Scale bars, 20 μm.

Polarity of the Parthenogenetic *msi1* Embryo

In the *Arabidopsis* wt fertilized zygote, the first division is unequal and separates an apical cell that develops as the embryo proper from a basal cell that forms the suspensor (Figure 3E). This apical-basal polarity remains active throughout embryo development and later marks the position of the shoot (apical) and of the root (basal) [14, 15]. We investigated evidence for apical-basal polarity after the first division of the parthenogenetic *msi1* zygote. During *msi1* egg cell elongation, the nucleus migrates to the apical pole (Figure 1B; n = 3) and undergoes mitosis at this location with the division plane perpendicular to the apical-basal axis as during the first wt zygotic division (Figures S2K and S2L; n = 4). The first division is unequal in 77% of divided parthenogenetic zygotes and leads to a smaller apical cell and a longer basal cell (Figure 3F; n = 86). In 37% parthenogenetic *msi1* embryos, PIN7-GUS expression is located in basal cells as in wt fertilized embryos (Figures 3C and 3D; n = 102). The initial apical-basal pattern typical of the wt single-cell embryo appears to be preserved in the parthenogenetic *msi1* embryo. This observation strongly suggests that the paternal copy of the genome is dispensable for establishment of the apical-basal polarity of the zygote in plants. Either the zygotic expression of the maternal copy of the genome is sufficient or polarity cues could be directly maternally inherited from the egg cell, which shows a polarized cytological organization [5, 16]. Genetic evidences for a maternal control of zygotic polarity are scarce [14] because mutants with altered apical-basal polarity in early embryogenesis have only provided evidence for a zygotic control [17, 18]. However, embryos obtained from unreduced embryo sacs in apomicts also show polarity, which indicates that the maternal genome is sufficient to sustain embryo development in asexual plant reproduction [2]. Alternatively, zygotic polarity could be provided by the surrounding maternal tissues of the ovule as suggested in the case of development of adventitious embryo sacs from unreduced cells in the ovule in aposporous apomicts [2].

Conclusion

Mutations in *MSI1* identify in plants a genetic pathway that allows production of a parthenogenetic embryo surrounded by autonomous endosperm, hence bypassing double fertilization. MSI1 is a member of the conserved FIS *Polycomb* group complex belonging to the PRC2 type [3, 19]. *Polycomb* group complexes regulate cell memory [20] and could control the timing of the female gametes maturation and arrest. Because the penetrance of loss of function in *FIS* genes on autono-

Table 1. Expression of Identity Markers in Autonomous Endosperm and Parthenogenetic Embryo in Comparison to WT

Marker Line	WT Unfertilized Central Cell	WT Fertilized Endosperm	Autonomous *msi1* Endosperm	WT Fertilized Embryo	Parthenogenetic *msi1* Embryo
MO11	+	+	+ (94)	—	– (94)
pMEA::GUS	+	+	+ (>100)	—	– (>100)
KS117	—	+	+ (>100)	—	– (>100)
KS22	—	+	+ (51)	—	– (51)
N9319	—	+	+ (91)	—	– (91)
PIN7-GUS	—	—	—	16% (405 embryos)	4.1% (2487 seeds)
G222	—	—	—	45% (426 embryos)	11% (1080 seeds)

In wt seeds, MO11 and pMEA::GUS markers are expressed in the central cell and in the syncytial endosperm, KS117, KS22, and N9319 markers are specifically expressed in the syncytial endosperm, and G222 and PIN7-GUS are markers of early embryonic development. In the autonomous seeds produced in the absence of fertilization, the same specificity of expression is observed. Numbers of 5 day-old autonomous seeds scored for each marker are indicated in parentheses for endosperm markers.

mous endosperm development is the highest for *msi1* [3, 13], it is possible that the egg cell is able to undergo parthenogenetic development in all *fis* mutants but with a very low penetrance as suggested by earlier reports [11]. Alternatively, the autonomous development of the central cell and of the egg cell in *msi1* could result from a deregulation of the cell cycle arrest of each of the plant gametes. According to results obtained in other species, MSI1 potentially interacts with the Retinoblastoma orthologs [21], and these proteins could associate in a complex with FIE [3, 22]. Autonomous endosperm development as been reported in *rbr1* mutant associated to overproliferation of other embryo sacs cells potentially including the egg cell [12]. In *Arabidopsis* wild-type embryo sacs, the RBR1/MSI1/FIE complex could be essential to control RBR1-dependent inhibition of activation of E2F-responsive genes and entry in S phase. Such a complex would thus control female gamete arrest and reinitiation of cell cycle at fertilization in plants.

Experimental Procedures

Plant Material and Growth Conditions

The KS117, KS22, MO11, N9104, N9312, and N9319 enhancer-trap lines expressing the GFP reporter protein were generated in J. Haseloff's lab (http://www.plantsci.cam.ac.uk/haseloff/home.html) (C24 ecotype). KS117 and KS22 were previously described [23, 24]. The pMEA::GUS reporter line (Ler ecotype) was kindly provided by Dr. A. Chaudhury [25]. The G222 enhancer-trap line and the PIN7-GUS were generously given by Prof. G. Jürgens and Dr. J. Friml. We previously isolated the *msi1-2* mutant line (JF2973) [13]. Plants were grown as previously described [13]. For crosses and autonomous seed development, anthers were removed 1.5 days before anthesis under greenhouse conditions. Anthesis corresponds to the time when flowers open and self-pollination occurs in *Arabidopsis*. Pollination of emasculated pistils was done at the forecasted day of anthesis.

Expression and Immunolocalization Analyses

Endosperm nuclei preparation for centromeres immunolocalization was modified from Lauber et al. [26]. Microtubule Stabilizing Buffer was replaced with 1× Phosphate Buffered Saline (PBS). Anti-HTR12 antibody was provided by Dr. P. Talbert [27] and diluted 1:2,000 in PBS. For secondary detection, we used an Alexa Fluor 488 goat anti-rabbit antibody (Molecular Probes, Invitrogen) diluted 1:100 in PBS. Antibody incubation was performed at room temperature for 1 hr. The nuclei were treated for 30 min at 37°C with 2 mg/mL RNase A (Sigma). DNA was stained with 1 μg/ml propidium iodide (Sigma) for 10 min. Mounting was done in Vectashield (Vector Laboratories). Because we did not succeed in removal of parthenogenetic embryos from *msi1* autonomous seeds, immunolocalization of centromeres could not be performed for this material, and we had to identify dividing cells in parthenogenetic embryos stained with Feulgen protocol [13] in order to count the total number of chromosomes.

Microscopy and Image Processing

Clearing and Nomarski microscopy was performed as described [13, 24]. For confocal microscopy, seeds were prepared with Feulgen staining [13]. We used a Zeiss LSM 510 microscope with a ×40 Plan-Apochromat (n.a. 1.0) or a ×63 Plan-Apochromat (n.a. 1.4) oil immersion objective. Serial optical sections were recorded with 0.4 μm to 0.6 μm depth. All figures were composed with Adobe Photoshop 5.5 (Adobe). For GFP expression pattern analysis, seeds were mounted in 0.3% plant agar (Duchefa) and observed under the confocal microscope. GUS staining was performed as previously described [23].

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/8/750/DC1/.

Acknowledgments

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